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(54) Title: FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF (57) Abstract The present invention is directed to novel fluorescent proteins from non-bioluminescent organisms from the Class Anthozoa. Also disclosed are cDNAs encoding the fluorescent proteins.		

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FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES
OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND
5 USES THEREOF

10 BACKGROUND OF THE INVENTION

Cross-reference to Related Application

This is a divisional application of U.S.S.N. 09/210,330 filed
15 on December 11, 1998.

Field of the Invention

This invention relates to the field of molecular biology.
More specifically, this invention relates to novel fluorescent proteins,
20 cDNAs encoding the proteins and uses thereof.

Description of the Related Art

Fluorescence labeling is a particularly useful tool for
marking a protein, cell, or organism of interest. Traditionally, a
25 protein of interest is purified, then covalently conjugated to a
fluorophore derivative. For *in vivo* studies, the protein-dye complex is
then inserted into cells of interest using micropipetting or a method of
reversible permeabilization. The dye attachment and insertion steps,
however, make the process laborious and difficult to control. An

alternative method of labeling proteins of interest is to concatenate or fuse the gene expressing the protein of interest to a gene expressing a marker, then express the fusion product. Typical markers for this method of protein labeling include β -galactosidase, firefly luciferase and bacterial luciferase. These markers, however, require exogenous substrates or cofactors and are therefore of limited use for *in vivo* studies.

A marker that does not require an exogenous cofactor or substrate is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a protein with an excitation maximum at 395 nm, a second excitation peak at 475 nm and an emission maximum at 510 nm. GFP is a 238-amino acid protein, with amino acids 65-67 involved in the formation of the chromophore.

Uses of GFP for the study of gene expression and protein localization are discussed in detail by Chalfie et al. in *Science* 263 (1994), 802-805, and Heim et al. in *Proc. Nat. Acad. Sci.* 91 (1994), 12501-12504. Additionally, Rizzuto et al. in *Curr. Biology* 5 (1995), 635-642, discuss the use of wild-type GFP as a tool for visualizing subcellular organelles in cells, while Kaether and Gerdes in *Febs Letters* 369 (1995), 267-271, report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng in *Febs Letters* 369 (1995), 331-334, while GFP expression in *Drosophila* embryos is described by Davis et al. in *Dev. Biology* 170 (1995), 726-729.

Crystallographic structures of wild-type GFP and the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel (Ormö et al., *Science* 273 (1996), 1392-1395; Yang, et al., *Nature Biotechnol* 14 (1996), 1246-1251). The barrel consists of beta sheets in a compact structure, where, in the center, an alpha helix containing

the chromophore is shielded by the barrel. The compact structure makes GFP very stable under diverse and/or harsh conditions such as protease treatment, making GFP an extremely useful reporter in general. However, the stability of GFP makes it sub-optimal for
5 determining short-term or repetitive events.

A great deal of research is being performed to improve the properties of GFP and to produce GFP reagents useful and optimized for a variety of research purposes. New versions of GFP have been developed, such as a "humanized" GFP DNA, the protein product of
10 which has increased synthesis in mammalian cells (Haas, et al., *Current Biology* 6 (1996), 315-324; Yang, et al., *Nucleic Acids Research* 24 (1996), 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue-, cyan- and yellow-green light emitting versions. Despite the great
15 utility of GFP, however, other fluorescent proteins with properties similar to or different from GFP would be useful in the art. Novel fluorescent proteins result in possible new colors, or produce pH-dependent fluorescence. Other benefits of novel fluorescent proteins include fluorescence resonance energy transfer (FRET) possibilities
20 based on new spectra and better suitability for larger excitation.

The prior art is deficient in novel fluorescent proteins wherein the DNA coding sequences are known. The present invention fulfills this long-standing need in the art.

25

SUMMARY OF THE INVENTION

The present invention is directed to DNA sequences encoding fluorescent proteins selected from the group consisting of:
(a) an isolated DNA from an organism from the Class Anthozoa which

encodes a fluorescent protein; (b) an isolated DNA which hybridizes to the isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to the degeneracy of the genetic code and that
5 encodes a fluorescent protein. Preferably, the DNA is isolated from a non-bioluminescent organism from Class Anthozoa. More preferably, the DNA has the sequence selected from the group consisting of SEQ ID Nos. 55 and 57, and the fluorescent protein has the amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58.

10 In another embodiment of the present invention, there is provided a vector capable of expressing the DNA of the present invention in a recombinant cell comprising said DNA and regulatory elements necessary for expression of the DNA in the cell. Preferably, the DNA encodes a fluorescent protein having the amino acid sequence
15 selected from the group consisting of SEQ ID Nos. 56 and 58.

In still another embodiment of the present invention, there is provided a host cell transfected with a vector of the present invention, such that the host cell expresses a fluorescent protein. Preferably, the cell is selected from the group consisting of bacterial
20 cells, mammalian cells, plant cells, insect cells and yeast cells

The present invention is also directed to an isolated and purified fluorescent protein coded for by DNA selected from the group consisting of: (a) isolated DNA from an organism from Class Anthozoa which encodes a fluorescent protein; (b) isolated DNA which hybridizes
25 to the isolated DNA of (a) and which encodes a fluorescent protein; and (c) isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to the degeneracy of the genetic code, and which encodes a fluorescent protein. Preferably, the protein has the amino

acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58.

The present invention is also directed to a DNA sequence encoding a fluorescent protein selected from the group consisting of:

5 (a) an isolated DNA which encodes a fluorescent protein, wherein said DNA is from an organism from Class Anthozoa and wherein said organism does not exhibit bioluminescence; (b) an isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of

10 (a) and (b) in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein. Preferably, the organism is from Sub-class Zoantharia, Order Zoanthidea. More preferably, the organism is from Sub-order Brachycnemina. Even more preferably, the organism is from Family Zoanthidae, Genus Zoanthus. Most

15 particularly, the present invention is drawn to a novel fluorescent protein from *Zoanthus* sp., zFP538.

The present invention is further directed to an amino acid sequence which can be used as a basis for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by

20 means of hybridization, wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14. Preferably, such an oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16.

Other and further aspects, features, and advantages of the

25 present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the modified strategy of 3'-RACE used to isolate the target fragments. Sequences of the oligonucleotides used are shown in Table 2. Dp1 and Dp2 are the degenerate primers used in the first and second PCR, respectively (see Tables 3 and 4 for the sequences of degenerate primers). In the case of *Zoanthus sp.*, the first degenerate primer used was NGH (SEQ ID No. 4), and the second degenerate primer used was GEGa (SEQ ID No. 6).

10

Figure 2 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus sp.*, zFP538.

Figure 3 shows the excitation and emission spectrum of mutant zFP538, i.e., m128v.

Figure 4 shows transient expression of pYNFP m128v-N1 and pEYFP-N1 in 293 cells, respectively. pYNFP m128v-N1 (Figure 4B) shows as bright fluorescent intensity as pEYFP-N1 (Figure 4A) by fluorescent microscopy. The fluorescence showed is green (pseudocolor), however, the actual color should be yellow.

Figure 5 shows that fusion protein PKC- γ -YNFP (M128V) translocated from cytosol to the plasma membrane when cells were treated with PMA (Phorbol 12-Myristate 13-Acetate). Figure 5A shows the result from control (without the treatment) and Figure 5B shows the result from PMA-treated cells.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *Aequorea victoria* GFP (SEQ ID No. 54) has been disclosed in Prasher et al., *Gene* 111 (1992), 229-33.

As used herein, the term "EGFP" refers to mutant variant of GFP having two amino acid substitutions: F64L and S65T (Heim et al., Nature 373 (1995), 663-664). The term "humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for expression of the protein in human cells (Yang et al., *Nucleic Acids Research* 24 (1996), 4592-4593).

As used herein, the term "YFP" refers to yellow fluorescent protein, and the term "EYFP" refers to enhanced yellow fluorescent protein.

As used herein, the term "NFP" refers to novel fluorescent protein, and the term "YNFP" refers to yellow novel fluorescent protein. Specifically, "YNFP" refers to zFP538

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells and Enzymes"

(IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about
5 the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not
10 limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed
15 under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA
20 sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination sequence may be located 3' to the coding sequence.

As used herein, the term "hybridization" refers to the process of association of two nucleic acid strands to form an
25 antiparallel duplex stabilized by means of hydrogen bonding between residues of the opposite nucleic acid strands.

The term "oligonucleotide" refers to a short (under 100 bases in length) nucleic acid molecule.

"DNA regulatory sequences", as used herein, are transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it

is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of
5 cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not
10 found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, heterologous DNA includes coding sequence in a construct where
15 portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "reporter gene" refers to a coding
20 sequence attached to heterologous promoter or enhancer elements and whose product may be assayed easily and quantifiably when the construct is introduced into tissues or cells.

The amino acids described herein are preferred to be in the "L" isomeric form. The amino acid sequences are given in one-letter
25 code (A: alanine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophan; Y: tyrosine; X: any residue). NH₂ refers to the free amino group present at the amino

terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243 (1969), 3552-59 is used.

5 The present invention is directed to an isolated DNA selected from the group consisting of: (a) isolated DNA from an organism from the Class Anthozoa which encodes a fluorescent protein; (b) isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) isolated DNA differing
10 from the isolated DNAs of (a) and (b) in codon sequence due to the degeneracy of the genetic code, and which encodes a fluorescent protein. Preferably, the DNA has the sequence selected from the group consisting of SEQ ID Nos. 55 and 57, and the fluorescent protein has the amino acid sequence selected from the group consisting of SEQ ID
15 Nos. 56 and 58. More preferably, the DNA is zFP538 or M128V.

 In another embodiment of the present invention, there is provided a vector capable of expressing the DNA of the present invention in a recombinant cell comprising said DNA and regulatory elements necessary for expression of the DNA in the cell. Specifically,
20 the DNA encodes a fluorescent protein having the amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58. Preferably, the vector is constructed by amplifying the DNA and then inserting the amplified DNA into EGFP-N1 backbone, or by fusing different mouse ODC degradation domains such as d1, d2 and d376 to
25 the C-terminal of the DNA and then inserting the fusion DNA into EGFP-N1 backbone.

 In still another embodiment of the present invention, there is provided a host cell transfected with the vector of the present invention, which expresses a fluorescent protein of the present

invention. Preferably, the cell is selected from the group consisting of bacterial cells, mammalian cells, plant cells, insect cells and yeast cells. A representative example of mammalian cell is HEK 293 cell and an example of bacterial cell is an *E. coli* cell.

5 The present invention is also directed to a DNA sequence encoding a fluorescent protein selected from the group consisting of: (a) an isolated DNA which encodes a fluorescent protein, wherein said DNA is from an organism from Class Anthozoa and wherein said organism does not exhibit bioluminescence; (b) an isolated DNA which
10 hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein. Preferably, the organism is from Sub-class Zoantharia, Order Zoanthidea. More preferably, the
15 organism is from Sub-order Brachycnemina. Even more preferably, the organism is from Family Zoanthidae, Genus Zoanthus.

 The present invention is also directed to an isolated and purified fluorescent protein coded for by DNA selected from the group consisting of: (a) an isolated protein encoded by a DNA which encodes
20 a fluorescent protein wherein said DNA is from an organism from Class Anthozoa and wherein said organism does not exhibit bioluminescence; (b) an isolated protein encoded by a DNA which hybridizes to isolated DNA of (a); and (c) an isolated protein encoded by a DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to
25 degeneracy of the genetic code. Preferably, the isolated and purified fluorescent protein is zFP538.

 The present invention is further directed to an amino acid sequence which can be used as a basis for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by

means of hybridization, wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14. Preferably, such an oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16 and is used as
5 a primer in polymerase chain reaction. Alternatively, it can be used as a probe for hybridization screening of the cloned genomic or cDNA library.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to
10 limit the present invention in any fashion.

EXAMPLE 1

15 **Biological Material**

Novel fluorescent proteins were identified from several genera of Anthozoa which do not exhibit any bioluminescence but have fluorescent color as observed under usual white light or ultraviolet light. Six species were chosen (see Table 1).

20

TABLE 1Anthozoa Species Used in This Study

Species	Area of Origination	Fluorescent Color
Anemonia majano	Western Pacific	bright green tentacle tips
Clavularia sp.	Western Pacific	bright green tentacles and oral disk
Zoanthus sp.	Western Pacific	green-yellow tentacles and oral disk
Discosoma sp. "red"	Western Pacific	orange-red spots oral disk
Discosoma striata	Western Pacific	blue-green stripes on oral disk
Discosoma sp. "magenta"	Western Pacific	faintly purple oral disk
Discosoma sp. "green"	Western Pacific	green spots on oral disk
Anemonia sulcata	Mediterranean	purple tentacle tips

EXAMPLE 2**cDNA Preparation**

Total RNA was isolated from the species of interest according to the protocol of Chomczynski and Sacchi (Chomczynski P., et al., *Anal. Biochem.* 162 (1987), 156-159). First-strand cDNA was synthesized starting with 1-3 µg of total RNA using SMART PCR cDNA synthesis kit (CLONTECH) according to the provided protocol with the only alteration being that the "cDNA synthesis primer" provided in the kit was replaced by the primer TN3 (5'- CGCAGTCGACCG(T)₁₃, SEQ ID No. 1) (Table 2). Amplified cDNA samples were then prepared as described in the protocol provided except the two primers used for PCR were the TS primer (5'-AAGCAGTGGTATCAACGCAGAGT, SEQ ID No. 2) (Table 2) and the TN3 primer (Table 2), both in 0.1 µM concentration. Twenty to twenty-five PCR cycles were performed to amplify a cDNA sample. The amplified cDNA was diluted 20-fold in water and 1 µl of this dilution was used in subsequent procedures.

TABLE 2

Oligos Used in cDNA Synthesis and RACE

5 TN3: 5'-CGCAGTCGACCG(T)₁₃
(SEQ ID No. 1)

T7-TN3: 5'-GTAATACGACTCACTATAGGGCCGCAGTCGACCG(T)₁₃
(SEQ ID No. 17)

10

TS-primer: 5'-AAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 2)

15 T7-TS:
5'-GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 18)

T7: 5'-GTAATACGACTCACTATAGGGC
(SEQ ID No. 19)

20

TS-oligo 5'-AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG
(SEQ ID No. 53)

25

EXAMPLE 3**Oligo Design**

To isolate fragments of novel fluorescent protein cDNAs,
5 PCR using degenerate primers was performed. Degenerate primers
were designed to match the sequence of the mRNAs in regions that
were predicted to be the most invariant in the family of fluorescent
proteins. Four such stretches were chosen (Table 3) and variants of
degenerate primers were designed. All such primers were directed to
10 the 3'-end of mRNA. All oligos were gel-purified before use. Table 2
shows the oligos used in cDNA synthesis and RACE.

TABLE 3

Key Amino Acid Stretches and Corresponding Degenerate Primers Used
for Isolation of Fluorescent Proteins

5

Stretch Position according to A. victoria GFP (7)	Amino Acid Sequence of the Key Stretch	Degenerated Primer Name and Sequence
20-25	GXVNGH (SEQ ID No. 3)	NGH: 5'- GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA (SEQ ID No. 4)
31-35	GEGEG (SEQ ID No. 5) GEGNG (SEQ ID No. 8)	GEGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG (SEQ ID No. 6) GEGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG (SEQ ID No. 7) GNGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG (SEQ ID No. 9) GNGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG (SEQ ID No. 10)
127-131	GMNFP (SEQ ID No. 11) GVNFP (SEQ ID No. 12)	NFP: 5' TTC CA(C,T) GGT (G,A)TG AA(C,T) TT(C,T) CC (SEQ ID NO. 13)
134-137	GPVM (SEQ ID No. 14)	PVMa: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG (SEQ ID NO. 15) PVMb: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG (SEQ ID NO. 16)

EXAMPLE 4Isolation of 3'-cDNA Fragments of nEPs

The modified strategy of 3'-RACE was used to isolate the
5 target fragments (see Figure 1). The RACE strategy involved two
consecutive PCR steps. The first PCR step involved a first degenerate
primer (Table 4) and the T7-TN3 primer (SEQ ID No. 17) which has a 3'
portion identical to the TN3 primer used for cDNA synthesis (for
sequence of T7-TN3, Table 2). The reason for substituting the longer
10 T7-TN3 primer in this PCR step was that background amplification
which occurred when using the shorter TN3 primer was suppressed
effectively, particularly when the T7-TN3 primer was used at a low
concentration (0.1 μ M) (Frohman et al., (1998) *PNAS USA*, 85, 8998-
9002). The second PCR step involved the TN3 primer (SEQ ID No. 1,
15 Table 2) and a second degenerate primer (Table 4).

TABLE 4

Combinations of Degenerate Primers for First and Second PCR Resulting in Specific Amplification of 3'-Fragments of nFP cDNA

Species	First Degenerate Primer	Second Degenerate Primer
Anemonia majano	NGH (SEQ ID No. 4)	GNGb (SEQ ID No. 10)
Clavularia sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Zoanthus sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Discosoma sp. "red"	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6), NFP (SEQ ID No. 13) or PVMb (SEQ ID No. 16)
Discosoma striata	NGH (SEQ ID No. 4)	NFP (SEQ ID No. 13)
Anemonia sulcata	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6) or NFP (SEQ ID No. 13)

5

The first PCR reaction was performed as follows: 1 µl of 20-fold
 10 dilution of the amplified cDNA sample was added into the reaction
 mixture containing 1X Advantage KlenTaq Polymerase Mix with
 provided buffer (CLONTECH), 200 µM dNTPs, 0.3 µM of first degenerate

primer (Table 4) and 0.1 μ M of T7-TN3 (SEQ ID No. 17) primer in a total volume of 20 μ l. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C, 1 min.; 72°C, 40 sec; 24 cycles for 95°C, 10 sec.; 62°C, 30 sec.; 72°C, 40 sec. The reaction was then diluted 20-fold in water and 1 μ l of this dilution was added to a second PCR reaction, which contained 1X Advantage KlenTaq Polymerase Mix with the buffer provided by the manufacturer (CLONTECH), 200 μ M dNTPs, 0.3 μ M of the second degenerate primer (Table 4) and 0.1 μ M of TN3 primer. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C (for GEG/GNG or PVM) or 52°C (for NFP), 1 min.; 72°C, 40 sec; 13 cycles for 95°C, 10sec.; 62°C (for GEG/GNG or PVM) or 58°C (for NFP), 30 sec.; 72°C, 40 sec. The product of PCR was cloned into PCR-Script vector (Stratagene) according to the manufacturer's protocol.

Different combinations of degenerate primers were tried in the first and second PCR reactions on the DNA from each species until a combination of primers was found that resulted in specific amplification--meaning that a pronounced band of expected size (about 650-800 bp for NGH and GEG/GNG and 350-500 bp for NFP and PVM--sometimes accompanied by a few minor bands) was detected on agarose gel after two PCR reactions. The primer combinations of choice for different species of the Class Anthozoa are listed in Table 4. Some other primer combinations also resulted in amplification of fragments of correct size, but the sequence of these fragments showed no homology to the other fluorescent proteins identified or to *Aequorea victoria* GFP.

EXAMPLE 5

Obtaining Full-Length cDNA Copies

Upon sequencing the obtained 3'-fragments of novel
5 fluorescent protein cDNAs, two nested 5'-directed primers were
synthesized for cDNA (Table 5), and the 5' ends of the cDNAs were
then amplified using two consecutive PCRs. In the next PCR reaction,
the novel approach of "step-out PCR" was used to suppress background
amplification. The step-out reaction mixture contained 1x Advantage
10 KlenTaq Polymerase Mix using buffer provided by the manufacturer
(CLONTECH), 200 μ M dNTPs, 0.2 μ M of the first gene-specific primer
(see Table 5), 0.02 μ M of the T7-TS primer (SEQ ID No. 18), 0.1 μ M of
T7 primer (SEQ ID No. 19) and 1 μ l of the 20-fold dilution of the
amplified cDNA sample in a total volume of 20 μ l. The cycling profile
15 was (Hybaid OmniGene Thermocycler, tube control mode): 23-27
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of
amplification was diluted 50-fold in water and one μ l of this dilution
was added to the second (nested) PCR. The reaction contained 1X
Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH),
20 200 μ M dNTPs, 0.2 μ M of the second gene-specific primer and 0.1 μ M
of TS primer (SEQ ID No. 2) in a total volume of 20 μ l. The cycling
profile was (Hybaid OmniGene Thermocycler, tube control mode): 12
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of
amplification was then cloned into pAtlas vector (CLONTECH) according
25 to the manufacturer's protocol.

TABLE 5Gene-Specific Primers Used for 5'-RACE

Species	First Primer	Second (Nested) Primer
Anemonia majano	5'-GAAATAGTCAGGCATACTGGT (SEQ ID No. 20)	5'-GTCAGGCATAC TGGTAGGAT (SEQ ID No. 21)
Clavularia sp.	5'-CTTGAAATAGTCTGCTATATC (SEQ ID No. 22)	5'-TCTGCTATATC GTCTGGGT (SEQ ID No. 23)
Zoanthus sp.	5'- GTTCTTGAAATAGTCTACTATGT (SEQ ID No. 24)	5'-GTCTACTATGTCTT GAGGAT (SEQ ID No. 25)
Discosoma sp. "red"	5'-CAAGCAAATGGCAAAGGTC (SEQ ID No. 26)	5'-CGGTATTGTGGCC TTCGTA (SEQ ID No. 27)
Discosoma striata	5'-TTGTCTTCTTCTGCACAAC (SEQ ID No. 28)	5'-CTGCACAACGG GTCCAT (SEQ ID No. 29)
Anemonia sulcata	5'-CCTCTATCTTCATTTCTGC (SEQ ID No. 30)	5'-TATCTTCATTTCT GCGTAC (SEQ ID No. 31)
Discosoma sp. "magenta"	5'-TTCAGCACCCCATCACGAG (SEQ ID No. 32)	5'-ACGCTCAGAGCTG GGTTCC (SEQ ID No. 33)
Discosoma sp. "green"	5'-CCCTCAGCAATCCATCACGTTC (SEQ ID No. 34)	5'-ATTATCTCAGTGGA TGGTTC (SEQ ID No. 35)

EXAMPLE 6

Expression of NFPs in *E. coli*

To prepare a DNA construct for novel fluorescent protein expression, two primers were synthesized for each cDNA: a 5'-directed "downstream" primer with the annealing site located in the 3'-UTR of the cDNA and a 3'-directed "upstream" primer corresponding to the site of translation start site (not including the first ATG codon) (Table 6). Primers with SEQ ID Nos. 41 and 42 were the primers used to prepare the zFP538 DNA. Both primers had 5'-heels coding for a site for a restriction endonuclease; in addition, the upstream primer was designed so as to allow the cloning of the PCR product into the pQE30 vector (Qiagen) in such a way that resulted in the fusion of reading frames of the vector-encoded 6xHis-tag and NFP. The PCR was performed as follows: 1 µl of the 20-fold dilution of the amplified cDNA sample was added to a mixture containing 1x Advantage KlenTaq Polymerase Mix with buffer provided by the manufacturer (CLONTECH), 200 µM dNTPs, 0.2 µM of upstream primer and 0.2 µM of downstream primer, in a final total volume of 20 µl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of this amplification step was purified by phenol-chlorophorm extraction and ethanol precipitation and then cloned into pQE30 vector using restriction endonucleases corresponding to the primers' sequence according to standard protocols.

All plasmids were amplified in XL-1 blue *E. coli* and purified by plasmid DNA miniprep kits (CLONTECH). The recombinant clones were selected by colony color, and grown in 3 ml of LB medium (supplemented with 100 µg/ml of ampicillin) at 37°C overnight. 100 µl

of the overnight culture was transferred into 200 ml of fresh LB medium containing 100 µg/ml of ampicillin and grown at 37°C, 200 rpm up to OD₆₀₀ 0.6-0.7. 1 mM IPTG was then added to the culture and incubation was allowed to proceed at 37°C for another 16 hours. The
5 cells were harvested and recombinant protein, which incorporated 6x His tags on the N-terminus, was purified using TALON™ metal-affinity resin according to the manufacturer's protocol (CLONTECH).

TABLE 6

Primers Used to Obtain Full Coding Region of nEPs for Cloning into Expression Construct

Species	Upstream Primer	Downstream Primer
Anemonia majano	5' -acatggatccgctctttcaaca agtttatac (SEQ ID No. 36) BamHI	5'-tagtactcgcgagcttattcgta tttcagtgaatac (SEQ ID No. 37) XhoI
Clavularia sp.	L: 5'-acatggatccaacattttttga gaaacg (SEQ ID No. 38) BamHI S: 5'-acatggatccaaagctctaacc accatg (SEQ ID No. 39) BamHI	5'-tagtactcgcgagcaacacaa accctcagacaa (SEQ ID No. 40) XhoI
Zoanthus sp.	5'- acatggatccgctcagtcacaag cacggt (SEQ ID No. 41) BamHI	5'-tagtactcgcgaggttgaactacat tcttatca (SEQ ID No. 42) XhoI
Discosoma sp. "red"	5'- acatggatccaggtcttccaagaat gttatac (SEQ ID No. 43) BamHI	5'-tagtactcgcgaggagccaagttc agcctta (SEQ ID No. 44) XhoI
Discosoma striata	5'- acatggatccagttggtccaagagtgtg (SEQ ID No. 45) BamHI	5'-tagcgcgagctctatcatgcctc gtcacct (SEQ ID No. 46) SacI
Anemonia sulcata	5'- acatggatccgcttccttttaagaagact (SEQ ID No. 47) BamHI	5'-tagtactcgcgagtccttgggagc ggcttg (SEQ ID No. 48) XhoI
Discosoma sp. "magenta"	5'- acatggatccagttgtccaagaatgtgat (SEQ ID No. 49) BamHI	5'-tagtactcgcaggccattacg ctaatac (SEQ ID No. 50) XhoI
Discosoma sp. "green"	5'-acatggatccagtgcaactaaagaagaaatg (SEQ ID No. 51)	5'-tagtactcgcgagattcggtttaata gccttg (SEQ ID No. 52)

EXAMPLE 7Novel Fluorescent Proteins and cDNAs Encoding the Proteins

One of the full-length cDNAs encoding novel fluorescent proteins is described herein (zFP538). The nucleic acid sequence and deduced amino acid sequence are SEQ ID Nos. 55 and 56, respectively. The spectral properties of zFP538 are listed in Table 7, and the emission and excitation spectrum for zFP538 is shown in Figure 2.

TABLE 7Spectral Properties of the Isolated zFP538

Species:	Zoanthus sp.	Max. Extinction Coefficient:	20,200
nFP Name:	zFP538	Quantum Yield	0.42
Absorbance Max. (nm):	528	Relative Brightness:*	0.38
Emission Max. (nm):	538		

*relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

EXAMPLE 8Construction of zFP538 Mutant

One mutant of zFP538 was generated, M128V. M128V was generated by introducing a wrong nucleotide in PCR during site-specific mutagenesis at position 65. One bright yellow colony was obtained, and the sequence of this clone was performed. It showed that this clone

contained wild type amino acid Lysine (K) at position 65, but had a substitution from Methionine (M) to Valine (V) at position 128 (numbering according to GFP). The nucleic acid sequence of M128V is shown in SEQ ID No. 57, and the deduced amino acid sequence is shown in SEQ ID No. 58.

Further investigations showed that M128V has spectral characteristics very similar to wild type protein zFP538 but folds much faster. Figure 3 shows the emission and excitation spectrum for M128V. Table 8 lists the spectral properties of M128V.

TABLE 8

Spectral Properties of the Isolated M128V

Species:	Zoanthus sp.	Max. Extinction Coefficient:	25,360
nFP Name:	M128V	Quantum Yield	0.43
Absorbance Max. (nm):	53	Relative Brightness:*	0.50
Emission Max. (nm):	540		

*relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

EXAMPLE 9

Construction and Functional Analysis of Vectors

Both wildtype (wt) and mutant zFP538 DNA were amplified via PCR and reconstructed to EGFP-N1 backbone. This vector has the

same multiple cloning sites as EGFP-N1. The nucleic acid sequence of pYNFPwt-N1 is shown in SEQ ID No. 59. The nucleic acid sequence of pYNFPM128V-N1 is shown in SEQ ID No. 60. Both pYNFPwt and pYNFPM128V keep the same multiple cloning sites as EGFP-N1.

5 Functional test of the generated vectors was performed by transient transfection in 293 cells. After 24-hour expression, pYNFPwt, pYNFPM12V and EYFP were compared side by side: pYNFPwt showed less fluorescent intensity than EYFP (data not shown); however, pYNFPM128V showed as bright fluorescent intensity as EYFP by
10 fluorescent microscopy (Figures 4A and 4B).

EXAMPLE 10

15 Generation of Destabilized zFP538 Vectors as Transcription Reporters

By using the same technology for destabilized EGFP, destabilized zFP538 vectors were constructed by fusing different mouse ODC degradation domains such as d1 and d2 to the C-terminal of zFP538. The d1 version of destabilized YNFP has three E to A mutations
20 within MODC degradation domain compared to d2 version. Vectors pYNFPM128V-MODCd1 and pYNFPM128V-MODCd2 were constructed in EGFP-N1 backbone.

25

EXAMPLE 11

Functional Analysis of Destabilized zFP538

Functional test of the destabilized zFP538 was performed by transient transfection in 293 cells. After 24-hour expression, the

fluorescent intensity was decreased gradually from d2 and d1 because of the fusion with different mouse ODC degradation domains. After 4-hour treatment with protein synthesis inhibitor cycloheximide, d2 fluorescent intensity did not change very much; however, d1
5 fluorescent intensity decreased further 50% of its original intensity. The half-life of d1 is around 4 hours.

M128V has fast folding and bright fluorescent intensity, which makes it useful for number of applications. Some fusion proteins were tested such as PKC-gamma-YNFP (M128V). PKC-gamma was
10 observed to translocate from cytosol to the plasma membrane when cells were treated with PMA (Phorbol 12-Myristate 13-Acetate) (Figures 5A and 5B).

15

EXAMPLE 12

Construction and Functional Test for Humanized M128V

Humanized M128V was generated, and then placed into the pEGFP-N1 backbone. This vector has the same mutiple cloning sites as
20 pEGFP-N1. Construction of C1 and pEGFP is in the process.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are incorporated by
25 reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects and ends inherent

therein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein, are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes
5 to the methods and compounds, and other uses, will occur to those skilled in the art and are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A DNA sequence encoding a fluorescent protein selected from the group consisting of:

5 (a) an isolated DNA which encodes a fluorescent protein, wherein said DNA is from an organism from a Class Anthozoa and wherein said organism does not exhibit bioluminescence;

(b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

10 (c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

15 2. The DNA sequence of claim 1, wherein said organism is from Sub-class Zoantharia.

3. The DNA sequence of claim 2, wherein said organism
20 is from Order Zoanthidea.

4. The DNA sequence of claim 3, wherein said organism is from Sub-order Brachycnemina.

25 5. The DNA sequence of claim 4, wherein said organism is from Family Zoanthidae.

6. The DNA sequence of claim 5, wherein said organism is from Genus Zoanthus.

5 7. A DNA sequence encoding a fluorescent protein selected from the group consisting of:

 (a) an isolated DNA which encodes a fluorescent protein having a nucleotide sequence selected from the group consisting of SEQ ID Nos. 55 and 57;

10 (b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

 (c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code, and which encodes a fluorescent protein.

15

8. The DNA sequence of claim 7, wherein said DNA encodes a fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58.

20

9. The DNA sequence of claim 7, wherein said DNA is non-humanized or humanized DNA.

25

10. The DNA sequence of claim 7, wherein said DNA is zFP538 or M128V.

11. A vector capable of expressing the DNA sequence of claim 1 in a recombinant cell, wherein said vector comprising said DNA and regulatory elements necessary for expression of the DNA in the cell.

5

12. The vector of claim 11, wherein said DNA encodes a fluorescent protein having the amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58.

10

13. The vector of claim 11, wherein said vector is constructed by amplifying said DNA and then inserting the amplified DNA into EGFP-N1 backbone.

15

14. The vector of claim 13, wherein said DNA is non-humanized or humanized DNA.

20

15. The vector of claim 13, wherein said DNA is zFP538 or M128V.

25

16. The vector of claim 11, wherein said vector is constructed by fusing different mouse ODC degradation domains to the C-terminal of said DNA and then inserting the fusion DNA into EGFP-N1 backbone.

17. The vector of claim 16, wherein said mouse ODC degradation domains are selected from the group consisting of d1, d2 and d376.

5

18. The vector of claim 16, wherein said DNA is non-humanized or humanized DNA.

10

19. The vector of claim 16, wherein said DNA is zFP538 or M128V.

20. A host cell transfected with the vector of claim 11,
15 wherein said cell is capable of expressing a fluorescent protein.

21. The host cell of claim 20, wherein said cell is selected from the group consisting of bacterial cells, mammalian cells, plant
20 cell, yeast and insect cells.

22. The host cell of claim 21, wherein said mammalian cell is HEK 293 cell.

25

23. The host cell of claim 21, wherein said bacterial cell is an *E. coli* cell.

24. An isolated and purified fluorescent protein coded for by DNA selected from the group consisting of:

(a) an isolated DNA which encodes a fluorescent protein
5 from an organism from Class Anthozoa, wherein said organism does not exhibit bioluminescence;

(b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

(c) an isolated DNA differing from the isolated DNAs of
10 (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

25. The isolated and purified fluorescent protein of claim
15 24, wherein said organism is from Sub-class Zoantharia.

26. The isolated and purified fluorescent protein of claim
20 25, wherein said organism is from Order Zoanthidea.

27. The isolated and purified fluorescent protein of claim
26, wherein said organism is from Sub-order Brachycnemina.

28. The isolated and purified fluorescent protein of claim
25 27, wherein said organism is from Family Zoanthidae.

29. The isolated and purified fluorescent protein of claim 28, wherein said organism is from Genus Zoanthus.

5 30. An isolated and purified fluorescent protein coded for by DNA selected from the group consisting of:

 (a) isolated DNA which encodes a fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58;

10 (b) isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

 (c) isolated DNA differing from said isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

15

 31. The isolated and purified fluorescent protein of claim 30, wherein said protein is zFP538.

20

 32. An amino acid sequence which can be used as a basis for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by means of hybridization, wherein said sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8,
25 11, 12, 14.

33. The amino acid sequence of claim 22, wherein said oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16

1/5

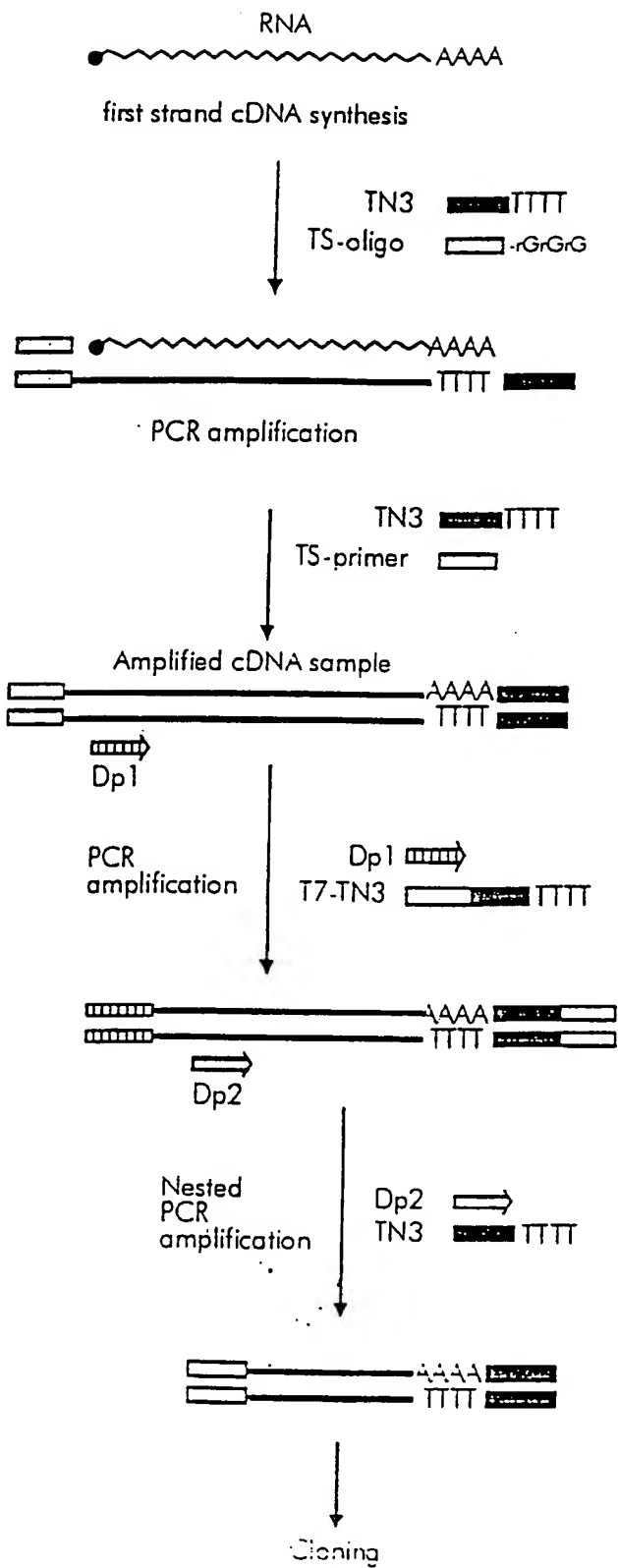


Figure 1

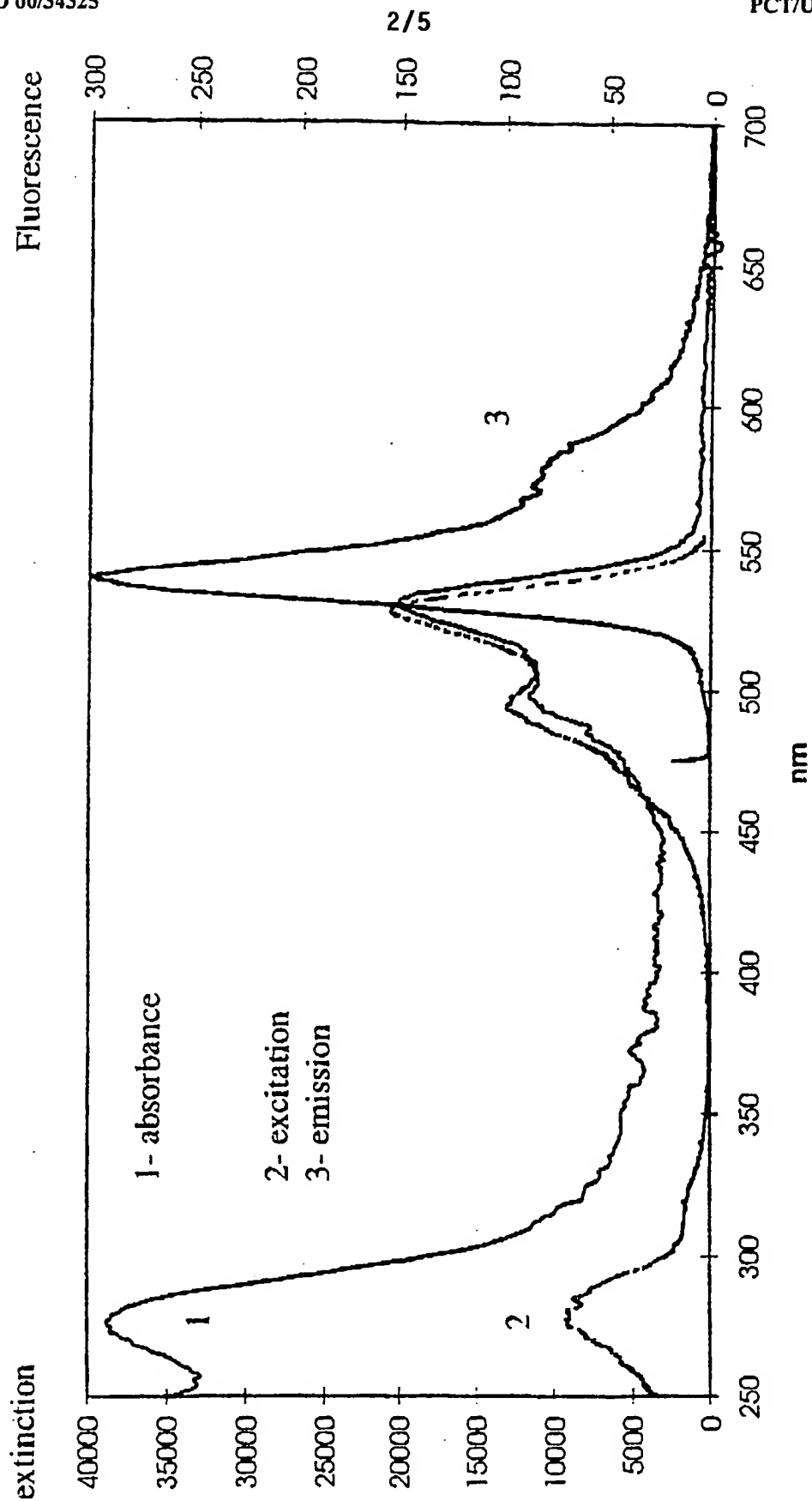


Figure 2

VFp4 - 4128 v mut
spectrum

Date:
20.08.99

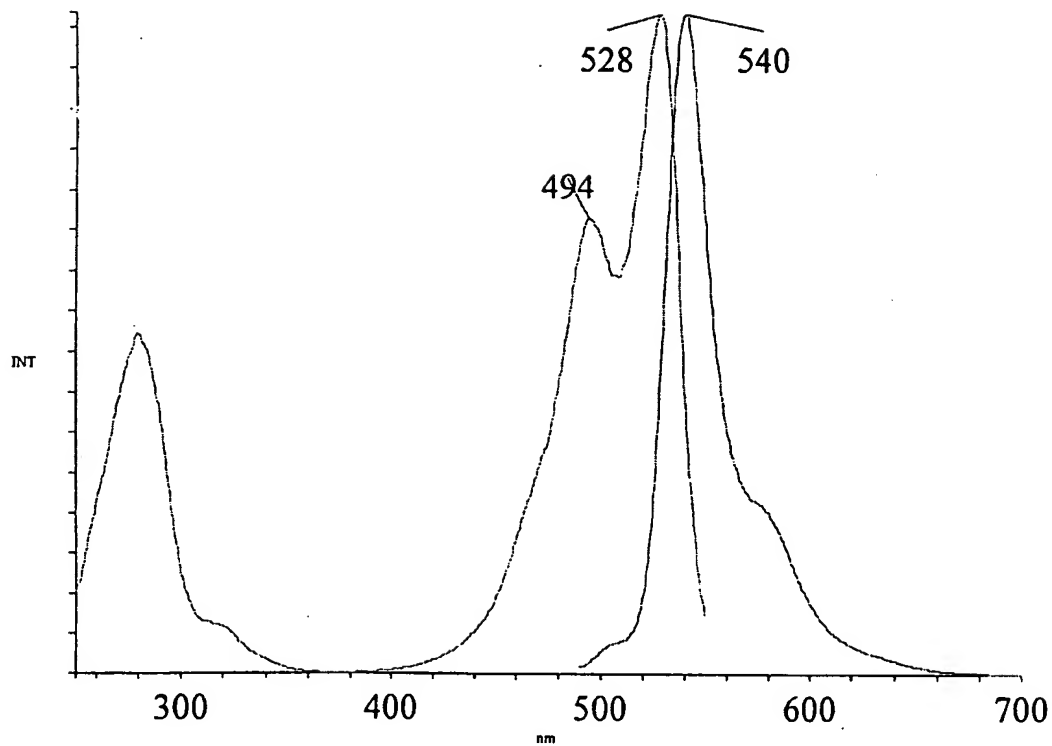
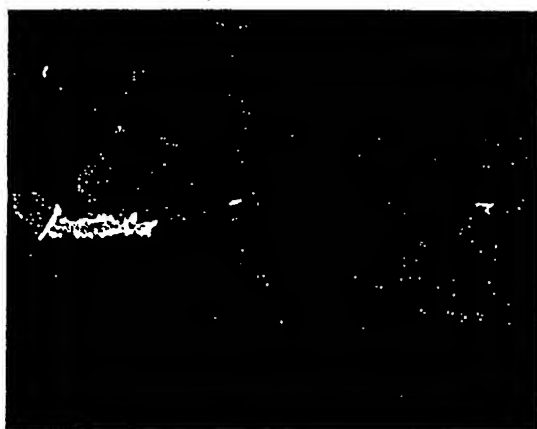


Figure 3

A. pEYFP-N1



B. pYNFP-N1 mut m128v

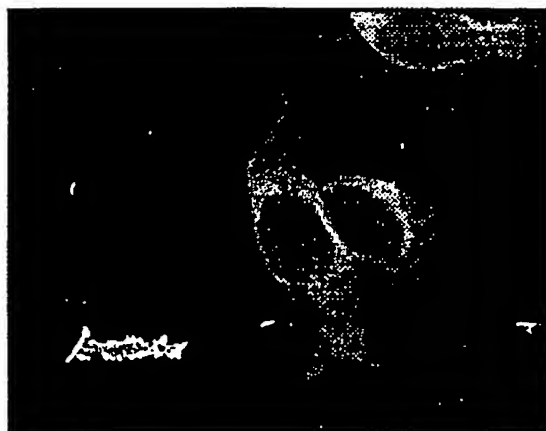


pseudocolor green. should be yellow

Figure 4

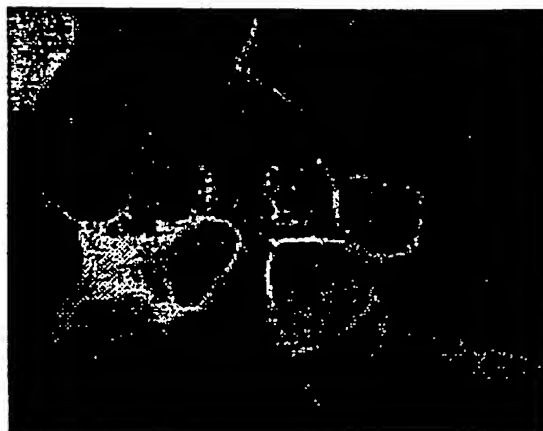
PKC ϵ -YFP translocation

A.



control

B.



PMA

Figure 5

SEQUENCE LISTING

<110> Lukyanov, Sergey A.
 Labas, Yulii A.
 Matz, Mikhail V.
 5 Fradkov, Arcady F.
 Fang, Yu
 Tan, Wenyan
 <120> Fluorescent proteins from non-bioluminescent
 species of Class Anthozoa, genes encoding such
 10 proteins and uses thereof
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5

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Discosoma striata
 5 <400> 28
 ttgtcttctt ctgcacaac 19
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Anemonia sulcata

<400> 31
tatcttcatt tcctgcgtac 20

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ttcagcaccc catcacgag 19

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acgctcagag ctgggttcc 19

25 <210> 34
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<400> 34

35 ccctcagcaa tccatcacgt tc 22

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<210> 36
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acatggatcc gctctttcaa acaagtttat c 31

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25 <212> DNA
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35 <211> 29
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of nFPs from *Clavularia sp.*
5 <400> 38
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<210> 39
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25 <223> downstream primer used to obtain full coding
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30 <210> 41
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of nFPs from *Zoanthus sp.*

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<210> 49
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<400> 50
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<210> 51
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of nFPs from *Discosoma sp.* "green"

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5 <210> 52

<211> 29

<212> DNA

<213> artificial sequence

<220>

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<223> downstream primer used to obtain full coding
region of nFPs from *Discosoma sp.* "green"

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<210> 53

<211> 33

<212> DNA

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20 <220>

<221> primer_bind

<223> TS-oligo used in cDNA synthesis and RACE

<400> 53

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25

<210> 54

<211> 238

<212> PRT

30 <213> *Aequorea victoria*

<220>

<223> amino acid sequence of GFP

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35

5

10

15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser

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	Gly	Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys
		35		40		45									
	Phe	Ile	Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu
5		50		55		60									
	Val	Thr	Thr	Phe	Ser	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro
		65		70		75									
	Asp	His	Met	Lys	Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu
		80		85		90									
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	Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn
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		140		145		150									
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		185		190		195									
	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	Ser	Lys	Asp
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	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	Val	Thr
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		230		235											

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<210> 55
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 <213> *Zoanthus* sp.

35

<220>
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 gttcgacaca gtttaciaaag caaagtctgt gccaaagtaag atgccggagt 650
 ggcacttcat ccagcataag ctctccctg aagaccgcag cgatgctaag 700
 15 aatcagaagt ggcagctgac agagcatgct attgcattcc cttctgcctt 750
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Tyr	His	Met	Glu	Gly	Cys	Val	Asn	Gly	His	Lys	Phe	Val	Ile	Thr	
				20					25					30	
Gly	Glu	Gly	Ile	Gly	Tyr	Pro	Phe	Lys	Gly	Lys	Gln	Thr	Ile	Asn	
				35					40					45	
Leu	Cys	Val	Ile	Glu	Gly	Gly	Pro	Leu	Pro	Phe	Ser	Glu	Asp	Ile	
				50					55					60	
35	Leu	Ser	Ala	Gly	Phe	Lys	Tyr	Gly	Asp	Arg	Ile	Phe	Thr	Glu	Tyr
				65					70					75	
	Pro	Gln	Asp	Ile	Val	Asp	Tyr	Phe	Lys	Asn	Ser	Cys	Pro	Ala	Gly

		80		85		90
	Tyr Thr Trp Gly Ser Phe Leu Phe Glu Asp Gly Ala Val Cys Ile					
		95		100		105
	Cys Asn Val Asp Ile Thr Val Ser Val Lys Glu Asn Cys Ile Tyr					
5		110		115		120
	His Lys Ser Ile Phe Asn Gly Met Asn Phe Pro Ala Asp Gly Pro					
		125		130		135
	Val Met Lys Lys Met Thr Thr Asn Trp Glu Ala Ser Cys Glu Lys					
		140		145		150
10	Ile Met Pro Val Pro Lys Gln Gly Ile Leu Lys Gly Asp Val Ser					
		155		160		165
	Met Tyr Leu Leu Leu Lys Asp Gly Gly Arg Tyr Arg Cys Gln Phe					
		170		175		180
	Asp Thr Val Tyr Lys Ala Lys Ser Val Pro Ser Lys Met Pro Glu					
15		185		190		195
	Trp His Phe Ile Gln His Lys Leu Leu Arg Glu Asp Arg Ser Asp					
		200		205		210
	Ala Lys Asn Gln Lys Trp Gln Leu Thr Glu His Ala Ile Ala Phe					
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20	Pro Ser Ala Leu Ala					
		230				

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	gatatccgtt caaagggaaa cagactatta atctgtgtgt gatcgaaggg		150
	ggaccattgc cattttccga agacatattg tcagctggct ttaagtacgg		200
	agacaggatt ttactgaat atcctcaaga catagtagac tatttcaaga		250
35	actcgtgtcc tgctggatat acatggggca ggtcttttct ctttgaggat		300
	ggagcagtct gcatatgcaa tgtagatata acagtgagtg tcaaagaaaa		350
	ctgcatttat cataagagca tattttaatgg agtgaatttt cctgctgatg		400

10

15

30

	170	175	180
Asp Thr Val Tyr	Lys Ala Lys Ser Val	Pro Ser Lys Met Pro	Glu
	185	190	195
Trp His Phe Ile	Gln His Lys Leu Leu	Arg Glu Asp Arg Ser	Asp
5	200	205	210
Ala Lys Asn Gln	Lys Trp Gln Leu Thr	Glu His Ala Ile Ala	Phe
	215	220	225
Pro Ser Ala Leu	Ala		
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20	cccaacgacc	ccgcccatt	gacgtcaata	atgacgtatg	ttcccatagt	150
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/29472

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07K 14/435; C12N 1/00, 1/15, 1/21, 5/10, 15/12, 15/63

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 252.3, 252.33, 325, 410, 254.11, 348, 369, 69.1; 530/350, 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	MATZ et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nature Biotechnology. October 1999, Volume 17, No. 10, pages 969-973, see entire document.	1-33
X, P	DE 197 18 640 A1 (WIEDENMANN) 22 July 1999, (22.07.99), see entire document.	24-25, 30
A	US 5,491,084 A (CHALFIE et al) 13 February 1996 (13.02.96).	24-25, 30
X	ANDERLUH et al. Cloning, sequencing, and expression of equinatoxin II. Biochemical and Biophysical Research Communications. 1996, Volume 220, No. 2, pages 437-442, see entire document.	1-2, 7, 11, 20-21, 23-25, 30



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

09 MARCH 2000

Date of mailing of the international search report

18 APR 2000

Name and mailing address of the ISA/US
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Box PCT
Washington, D.C. 20231

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29472

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — L	MACEK et al. Intrinsic tryptophan fluorescence of equinatoxin II, a pore-forming polypeptide from the sea anemone <i>Actinia equina</i> L, monitors its interaction with lipid membranes. European Journal of Biochemistry. 1995, Volume 234, pages 329-335, entire document. Cited as "L" document because it establishes fluorescence of equinatoxin II.	24-25, 30 1-2, 7, 11, 20-21, 23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/29472

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 7-10, 12, 14-15, 30-33
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Since the sequence listing (CRF) submitted by applicant is defective, a sequence search could not be performed. Accordingly, claims 7-10, 12, 14-15 and 30-33 were searched only in-part based on a word search.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/29472

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/320.1, 252.3, 252.33, 324, 410, 254.11, 348, 369, 69.1; 530/350; 536/23.5

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Dialog files 155, 5, 434, 34, 358, 28, 44, 77 (Medline, Biosis, Scisearch, Derwent Biotech Abs., Oceanic Abs., Aquatic & Fish Abs., Dissertation Abs. Online, Conference Papers Index); STN-CAS files registry, CAPLUS; WEST files USPT, Derwent WPI

search terms: fluoresc?, bioluminesc?, protein?, polypeptide?, gene#, anthozo?, zoanth?, brachycnem? coral?, cnidar?, anemon?, zfp538, mahskhglk/sqsp, vngh/sqep, gegeg/sqep, gegng/sqep, gmnfp/sqep, gvnfp/sqep, gpvn/sqep